

## **CCN3 COMPOSITIONS AND METHODS**

### **FIELD OF THE INVENTION**

[0001] The present invention relates to materials and methods involving extracellular matrix signaling molecules in the form of polypeptides involved in cellular responses to growth factors. More particularly, the invention is directed to CCN3-related peptides, compositions thereof, and methods of using these polypeptides. The invention is also directed to anti-CCN3 antibodies.

### **BACKGROUND**

[0002] Angiogenesis, or the formation of new blood vessels from pre-existing ones, is a complex process requiring the coordinated execution of multiple cellular events (45). The sprouting of vessels requires degradation of the basement membrane surrounding the parental vessel, migration of vascular endothelial cells towards the angiogenic stimulus, proliferation of endothelial cells and their alignment into tubular structures, and coalescence of new vessels into circular loops to provide blood supply to the target tissue (45). Angiogenesis is essential for embryogenesis, and in the adult, it is important in the female reproductive cycle and in wound healing. Angiogenesis may underlie a number of pathological conditions including diabetic retinopathy, arthritis, arteriosclerosis, psoriasis, and cancer (46). It is now clear that angiogenesis is regulated by a network of multiple inducers and inhibitors (47,48).

[0003] The CCN<sup>1</sup> family of matricellular proteins are cysteine-rich, secreted proteins that are associated with the ECM but serve regulatory rather than structural functions. Members of the CCN family, which include CCN1 (CYR61), CCN2 (CTGF), CCN3 (NOV), CCN4 (WISP-1),

CCN5 (WISP-2), and CCN6 (WISP-3)(3,4), are composed of an N-terminal secretory signal peptide followed by four conserved domains with homology to insulin-like growth factor binding protein, von Willebrand factor type C repeat, thrombospondin type 1 repeat, and a C-terminal domain (CT) with heparin-binding motifs and sequence similarity to the C-termini of von Willebrand factor and mucin (6). In keeping with their homology to ECM proteins and localization to the ECM, several CCN proteins have been shown to support cell adhesion, induce focal adhesion complexes and stimulate adhesive signaling (7-9).

[0004] Among members of the CCN family, CCN1 and CCN2 have been most extensively characterized. Both proteins stimulate cell migration, promote cell survival, and augment growth factor-induced mitogenesis (10-14). Both proteins are known to induce angiogenesis and chondrogenesis (12,15-18). Although CCN proteins do not contain a RGD sequence motif, both CCN1 and CCN2 are direct ligands of multiple integrin receptors, which mediate many of their activities (11,13,14,19-22). Targeted disruption of the *CCN1* gene in mice resulted in embryonic lethality due to vascular defects (23), whereas *CCN2*-null mice die perinatally due to respiratory failure as a consequence of skeletal malformation (18). These findings indicate that members of the CCN family serve essential and non-redundant functions during development.

[0005] Although CCN3 was first identified more than 10 years ago, little is known about its biochemical activities and biological functions. During embryonic development, CCN3 is widely expressed in derivatives of all three germ layers, with high levels of expression in skeletal muscle, smooth muscle of vessel walls, the nervous system, adrenal cortex, and differentiating chondrocytes (24-27). In the adult, CCN3 expression is high in the arterial vessel wall, and the expression pattern changes following vascular injury (28). CCN3 interacts with the epidermal growth factor-like domain of Notch1 and positively regulate Notch signaling (29). In addition,

CCN3 interacts with fibulin in a yeast two-hybrid assay and may regulate calcium signaling (30,31). Altered expression of CCN3 has been observed in a variety of tumors, including hepatocellular carcinomas, Wilm's tumors, Ewing's sarcomas, gliomas, rhabdomyosarcomas, and adrenocortical carcinomas (27,32-34).

[0006] CCN3 is demonstrated herein for the first time to be angiogenic, thereby being capable of exerting a concerted and coordinated influence on one or more of the particularized functions collectively characterizing such complex biological processes as angiogenesis and oncogenesis. Therefore, a need exists to develop therapeutics for modulating the biological role of CCN3. Moreover, a need exists to develop methods of treatment for modulating the biological role of CCN3 in patients with CCN3-mediated cancers.

#### SUMMARY

[0007] The present invention provides extracellular matrix (ECM) signaling molecule-related materials and methods. In particular, the present invention is directed to CCN3-related peptides, compositions thereof, and methods of using these polypeptides. The invention is also directed to anti-CCN3 antibodies.

[0008] One aspect of the present invention relates to a method of screening for a modulator of angiogenesis comprising contacting a test biological sample capable of undergoing angiogenesis with an ECM signaling molecule and a suspected modulator. As a control, a second biological sample is also contacted with an ECM signaling molecule. A modulator of angiogenesis is identified by the ability to alter the level of angiogenesis in the test sample. The ECM signaling molecule may be CCN3 or a fragment, variant, analog, homolog or a derivative thereof that

maintains as least one biological activity of CCN3. Another aspect of the present invention relates to a modulator of CCN3 activity identified by the present method.

[0009] Another aspect of the present invention relates to a method of screening for a modulator of angiogenesis comprising implanting a test implant into a test animal, wherein the test implant comprises a suspected modulator and an ECM signaling molecule. As a control, a second implant comprising an ECM signaling molecule is implanted into a test animal, which may be the same animal or a different test animal. A modulator of angiogenesis is identified by its ability to alter the level of blood vessel development in the test implant when compared to the control sample. The ECM signaling molecule may be CCN3 or a fragment, variant, analog, homolog or a derivative thereof that maintains as least one biological activity of CCN3. Another aspect of the present invention relates to a modulator of CCN3 activity identified by the present method.

[0010] Another aspect of the present invention relates to a method of screening for a modulator of oncogenesis comprising contacting a tumor with a suspected modulator along with an ECM signaling molecule. As a control, a second tumor is also contacted with an ECM signaling molecule. A modulator of oncogenesis may be identified by its ability to alter the level of oncogenesis of the test tumor when compared to the control tumor. The ECM signaling molecule may be CCN3 or a fragment, variant, analog, homolog or a derivative thereof that maintains as least one biological activity of CCN3. Another aspect of the present invention relates to a modulator of CCN3 activity identified by the present method.

[0011] Another aspect of the present invention relates to a method of screening for a modulator of cell adhesion comprising contacting a test biological sample on a surface compatible with cell adherence with a suspected modulator along with an ECM signaling molecule. As a control, a

second biological sample on a surface compatible with cell adherence is also contacted with an ECM signaling molecule. A modulator of cell adhesion is identified by its ability to alter the level of cell adhesion of the test sample when compared to the control sample. The ECM signaling molecule may be CCN3 or a fragment, variant, analog, homolog or a derivative thereof that maintains as least one biological activity of CCN3. Another aspect of the present invention relates to a modulator of CCN3 activity identified by the present method.

[0012] Another aspect of the present invention relates to a method of screening for a modulator of cell migration comprising seeding cells capable of undergoing cell migration onto a test gel matrix comprising a suspected modulator and an ECM signaling molecule. As a control, cells capable of undergoing cell migration are also seeded onto a second biological sample gel matrix comprising an ECM signaling molecule. A modulator of cell adhesion may be identified by its ability to alter the level of cell migration in the test matrix when compared to the control matrix. The ECM signaling molecule may be CCN3 or a fragment, variant, analog, homolog or a derivative thereof that maintains as least one biological activity of CCN3. Another aspect of the present invention relates to a modulator of CCN3 activity identified by the present method.

[0013] Another aspect of the present invention relates to a peptide that modulates the binding of CCN3 to an integrin selected from the group consisting of  $\alpha_v\beta_3$ ,  $\alpha_5\beta_1$  and  $\alpha_6\beta_1$ , or a variant, analog, homolog or derivative of said peptide.

[0014] Another aspect of the present invention relates to a composition comprising a peptide that modulates the binding of CCN3 to an integrin selected from the group consisting of  $\alpha_v\beta_3$ ,  $\alpha_5\beta_1$  and  $\alpha_6\beta_1$ , or a variant, analog, homolog or derivative of said peptide. In a related aspect, the composition may further comprise one or more peptides that modulate the binding of a CCN polypeptide to an integrin, or a variant, analog, homolog or derivative of said one or more

peptides, wherein said CCN polypeptide is selected from the group consisting of CCN1, CCN2, CCN4, CCN5 and CCN6.

[0015] Another aspect of the present invention relates to an antibody that modulates the binding of CCN3 to an integrin selected from the group consisting of  $\alpha_v\beta_3$ ,  $\alpha_5\beta_1$  and  $\alpha_6\beta_1$ , or a variant, analog, homolog or derivative of said peptide.

[0016] Another aspect of the present invention relates to a composition comprising an antibody that modulates the binding of CCN3 to an integrin selected from the group consisting of  $\alpha_v\beta_3$ ,  $\alpha_5\beta_1$  and  $\alpha_6\beta_1$ , or a variant, analog, homolog or derivative of said peptide. In a related aspect, the composition further comprises one or more antibodies that modulate the binding of a CCN polypeptide to an integrin, wherein said CCN polypeptide is selected from the group consisting of CCN1, CCN2, CCN4, CCN5 and CCN6.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0017] Fig. 1 demonstrates the purification of CCN3. Panel A: fractions (30  $\mu$ l per lane) from nickel-agarose column chromatography were separated by 10% SDS-PAGE, followed by staining with Coomassie Brilliant Blue (MW-molecular weight markers). Panel B: same fractions as in A (10  $\mu$ l per lane) were analyzed on SDS-PAGE followed by immunoblotting with anti-CCN3 antibodies.

[0018] Fig. 2 demonstrates the adhesion of HUVECs to CCN3. Panel A: HUVECs were plated in microtiter wells coated with the indicated amount of CCN3. After incubation at 37°C for 30 min, adherent cells were fixed, stained with methylene blue, and extracted dye was quantified by absorbance at 620 nm. Panel B: microtiter wells were coated with BSA, 12  $\mu$ g/ml CCN3, or 0.5  $\mu$ g/ml VN, and where indicated, they were blocked with affinity purified anti-CCN3 antibodies

or normal rabbit IgG for 1 h at 37°C prior to the addition of HUVECs. Panel C: cells were plated on microtiter wells coated with 12 µg/ml CCN3, 3 µg/ml FN or 0.5 µg/ml VN. EDTA (10 mM) was added alone or in combination with Ca<sup>2+</sup> or Mg<sup>2+</sup> (20 mM) as indicated. Panel D: cells were incubated with GRGDSP, GRGESP peptides (0.2 mM), heparin (1 µg/ml); or in combination prior to plating on microtiter wells coated with CCN3, FN or VN. Data shown are mean ± SD of triplicate determinations and are representative of three experiments.

[0019] Fig. 3 demonstrates that HUVEC adhesion to CCN3 is mediated through integrins  $\alpha_v\beta_3$ ,  $\alpha_6\beta_1$ , and  $\alpha_5\beta_1$ . Cell adhesion assays were performed with HUVECs as described in Fig. 2.

Panel A: cells were incubated with 40 µg/ml mAbs against integrins  $\alpha_6$  and  $\beta_1$  for 1 h prior to being plated on microtiter wells coated with 15 µg/ml CCN3, 0.5 µg/ml VN, or 5 µg/ml LN.

Panel B: cells were incubated with 40 µg/ml LM609 (anti- $\alpha_v\beta_3$  mAb) for 1 h prior to being plated on microtiter wells coated with 15 µg/ml CCN3, 0.25 µg/ml VN, or 3 µg/ml FN. Panel C: cells were incubated with 20 µg/ml anti- $\alpha_5\beta_1$  integrin antibody JBS5 for 1 h prior to being plated on microtiter wells coated with 15 µg/ml CCN3, 0.5 µg/ml VN, 1.5 µg/ml FN. Panel D: cells were incubated with a combination of mAbs against integrins  $\alpha_6$  (GoH3, 20 µg/ml),  $\alpha_v\beta_3$  (LM609, 20 µg/ml) and  $\alpha_5\beta_1$  (JBS5, 10 µg/ml) for 1 h prior to being plated on microtiter wells coated with 15 µg/ml CCN3, 0.25 µg/ml VN, 5 µg/ml LN, and 1.5 µg/ml FN. Data shown are mean ± SD of triplicate determinations and are representative of three experiments.

[0020] Fig. 4 demonstrates that CCN3 binds directly to integrin  $\alpha_v\beta_3$ . Panel A: microtiter wells were coated with purified integrin  $\alpha_v\beta_3$  (1 µg/ml) and blocked with 1% BSA. Binding of varying concentrations of CCN3 was detected using anti-CCN3 antibodies. Panels B-D: microtiter wells were coated with CCN3 (10 µg/ml) or VN (1 µg/ml) and blocked with BSA. Effects of pre-

incubation of coated proteins with anti-CCN antibodies (B), 20  $\mu\text{g/ml}$  LM609 (D), or 20  $\mu\text{g/ml}$  normal mouse IgG (D) prior to addition and binding of integrin  $\alpha_v\beta_3$  was observed. Panel C: integrin  $\alpha_v\beta_3$  was incubated with 5 mM EDTA, EDTA + 10 mM  $\text{Mg}^{2+}$ , 0.2 mM RGDS peptide, or 0.2 mM RGES peptide for 30 min at 4°C prior to addition into microtiter wells. Binding was detected using anti- $\alpha_v$  antibodies. Data shown are from three separate experiments and represented as mean  $\pm$  SD of duplicate determinations in each experiment.

[0021] Fig. 5 demonstrates that CCN3 binds directly to integrin  $\alpha_5\beta_1$ . Panel A: microtiter wells were coated with purified integrin  $\alpha_5\beta_1$  (1  $\mu\text{g/ml}$ ) and blocked with 1% BSA. Binding of varying concentrations of CCN3 was detected using anti-CCN3 antibodies. Panels B-D: microtiter wells were coated with CCN3 (10  $\mu\text{g/ml}$ ) or FN (10  $\mu\text{g/ml}$ ) and blocked with BSA. Effects of pre-incubation of coated proteins with anti-CCN antibodies (B), 20  $\mu\text{g/ml}$  JBS5 (D), or 20  $\mu\text{g/ml}$  normal mouse IgG (D) prior to binding of integrin  $\alpha_5\beta_1$  was observed. Panel C: integrin  $\alpha_5\beta_1$  was incubated with 5 mM EDTA, EDTA + 10 mM  $\text{Mg}^{2+}$ , 2 mM RGDS peptide, or 2 mM RGES peptide for 30 min at 4°C prior to addition into microtiter wells. Binding was detected using either anti- $\beta_1$  (B) or anti- $\alpha_5$  (C, D) antibodies. Data shown are from three separate experiments and represented as mean  $\pm$  SD of duplicate.

[0022] Fig. 6 demonstrates migration of BACEC to CCN3. BACEC migration was monitored using a modified Boyden chamber assay. Cells were added to wells in the lower chamber and allowed to attach to the gelatinized polycarbonate filter. bFGF (10 ng/ml), CCN3 (0.25  $\mu\text{g/ml}$  unless otherwise indicated), VN (10  $\mu\text{g/ml}$ ), or FN (10  $\mu\text{g/ml}$ ) were placed in the top chamber (unless otherwise indicated). Cells were allowed to migrate for 4 h at 37°C before being fixed and stained, and cells that migrated into the upper chamber were counted in ten random high



power fields. Cell migration in response to varying concentrations of CCN3 (A), the presence of anti-CCN3 antibodies (B), or 0.2 mM GRGDSP or GRDESP peptides (D) was assessed.

Panel C: the migration of BACEC was measured in a checkerboard-type analysis. CCN3 or bFGF were added to the upper chamber, the lower chamber, neither chamber, or both chambers as indicated. Data shown are mean  $\pm$  SD of triplicate determinations and are representative of three experiments.

[0023] Fig. 7 demonstrates that integrins  $\alpha_v\beta_3$  and  $\alpha_5\beta_1$  mediate migration of BACEC to CCN3.

Migration assays were performed using a modified Boyden chamber. As chemoattractants, CCN3 (0.25  $\mu$ g/ml), VN (10  $\mu$ g/ml), and FN (10  $\mu$ g/ml) were placed in the top chamber.

Panel A: cells were treated with anti-integrin  $\alpha_v$  (AV1, 60  $\mu$ g/ml) or anti-integrin  $\alpha_v\beta_3$  (LM609, 60  $\mu$ g/ml) for 1 h prior to chamber loading. Cells were also pre-incubated with either anti-integrin  $\alpha_5$  (SAM-1, 40  $\mu$ g/ml) (B), both SAM-1 (12.5  $\mu$ g/ml) and LM609 (25  $\mu$ g/ml) (C), or anti-integrin  $\alpha_6$  (GoH3, 25  $\mu$ g/ml) (D) for 1 h prior to chamber loading. Data shown are mean  $\pm$  SD of triplicate determinations and are representative of three experiments.

[0024] Fig. 8 demonstrates that CCN3 protects HUVECs from apoptosis. HUVECs were serum-starved prior to attachment to coverslips pre-coated with 20  $\mu$ g/ml LN. Panel A: cells were then incubated in serum free medium for 4h. This was followed by addition of serum, various concentrations of CCN3 protein or 50  $\mu$ M GRGDSP peptide and incubation at 37°C for an additional 16 h. Cells were fixed and apoptosis was monitored by using a TUNEL assay; the number of apoptotic cells were then counted. Where indicated, CCN3 was pre-incubated with anti-CCN3 antibodies prior to addition into the medium. Panel B: HUVECs treated as described in panel A were labeled with BrdUrd for 16 h, and percentages of cells incorporating label in the

absence or presence of CCN3 are shown. Data shown are mean  $\pm$  SD of triplicate determinations and are representative of three experiments.

[0025] Fig. 9 demonstrates that CCN3 induces neovascularization in rat corneas. Hydron pellets containing test substances were made and implanted into rat corneas (Table 1). Blood vessel formation was visualized by perfusion with colloidal carbon 7 days after implantation. Vessel formation due to Hydron pellets containing CCN3 storage buffer (A), bFGF (B), CCN3 protein (C), and CCN3 protein pre-incubated with anti-CCN3 antibodies (D), are shown.

#### DETAILED DESCRIPTION

[0026] In contrast to *CCN1* and *CCN2*, which are immediate-early genes transcriptionally activated by mitogenic growth factors in fibroblasts and are repressed under conditions of growth arrest (35-37), *CCN3* is repressed by growth factors but induced by serum deprivation or contact inhibition (38,39). Thus, it has been hypothesized that CCN3 may serve as an antagonist to CCN1 and CCN2, and play antithetical roles in similar biological processes. Since CCN1 and CCN2 have been shown to be angiogenic inducers (12,16,17), it has been speculated that CCN3 might work as an angiogenic inhibitor.

[0027] Surprisingly, we show below that purified CCN3 is capable of proangiogenic activities in endothelial cells. CCN3 is shown below to support endothelial cell adhesion, stimulates directed cell migration, and promotes cell survival (Figs. 2,6,8). Furthermore, CCN3 is shown below to induce neovascularization *in vivo* in a corneal micropocket assay (Fig. 9). These activities are inhibited by antibodies specific for CCN3, showing that they are intrinsic properties of the CCN3 polypeptide. These findings establish CCN3 as a novel integrin ligand and angiogenic inducer, provide insights into its mechanism of action, and suggest biological functions for CCN3 both in normal development and in pathological conditions where its aberrant expression has been

observed. In particular, CCN3 may be part of the network of multiple inducers and inhibitors that regulate angiogenesis.

[0028] The discovery that CCN3 is an angiogenic factor helps to shed light on its functions in development and disease. CCN3 is expressed in hypertrophic cartilage (26), where vessel growth is required for the formation of a scaffold onto which the osteoblasts settle and deposit bone matrix (49). Thus, CCN3-induced angiogenesis may be important in endochondral ossification. During nephrogenesis, CCN3 is localized to the metanephric mesenchyme into which endothelial cells are recruited (32). These endothelial cells then proliferate and form a capillary network as the metanephric mesenchyme develops to form the glomeruli, the basic units of filtration (50). The presence of CCN3 might help serve as a chemotactic and survival factor for endothelial cells. In addition, CCN3 expression is correlated with various tumors, including Wilm's tumors, and benign adrenocortical tumors (27,32,51). It is well established that tumor growth beyond ~1 mm in size requires the growth of new vessels to provide the necessary blood supply (47,52). Thus, the expression of CCN3 in tumors is consistent with its angiogenic activity. Furthermore, the Wilms' Tumor suppressor gene (WT1) was shown to negatively regulate CCN3 expression (53). It is possible to speculate that, as part of its function, WT1 down-regulates the angiogenic inducer CCN3 to help suppress tumor growth.

[0029] We have also identified the cell surface receptors that mediate CCN3 functions in endothelial cells. CCN3 is shown below to support endothelial cell adhesion through integrins  $\alpha_v\beta_3$ ,  $\alpha_5\beta_1$ , and  $\alpha_6\beta_1$ , whereas chemotaxis is mediated through  $\alpha_v\beta_3$  and  $\alpha_5\beta_1$  (Figs. 3 and 7). Furthermore, CCN3 is shown below to bind directly to integrins  $\alpha_v\beta_3$  and  $\alpha_5\beta_1$  (Figs. 4 and 5). Despite lacking a RGD sequence motif, CCN3 binds directly to integrins  $\alpha_v\beta_3$  and  $\alpha_5\beta_1$ .

[0030] It is well established that integrins are important in developmental and pathological angiogenesis (54). The role of integrin  $\alpha_5\beta_1$  in developmental angiogenesis has been established genetically in mice, where targeted gene disruptions in integrins  $\alpha_5$  or  $\beta_1$  resulted in embryonic lethality with prominent angiogenic defects (55-57). Inasmuch as CCN3 is shown below to act directly on endothelial cells through integrin  $\alpha_5\beta_1$  to promote cell adhesion and migration, the binding of CCN3 to  $\alpha_5\beta_1$  may be critical to CCN3-mediated angiogenesis. Interestingly, CCN3 is the only member of the CCN protein family known to bind integrin  $\alpha_5\beta_1$  to date. Integrin  $\alpha_5\beta_1$  plays no role in CCN1-mediated endothelial cell adhesion (7), and we have found that CCN1 does not bind purified  $\alpha_5\beta_1$  in a solid phase binding assay (data not shown).

[0031] A wealth of data also supports the notion that integrin  $\alpha_v\beta_3$  plays a critical role in angiogenesis. Importantly, antagonists of integrin  $\alpha_v\beta_3$  effectively block angiogenesis both *in vitro* and *in vivo*, and inhibit tumor formation in animal models (58,59). A humanized monoclonal antibody against integrin  $\alpha_v\beta_3$ , Vitaxin, is currently undergoing clinical trial as an anti-cancer drug (60). This therapeutic approach is predicated on the premise that integrin  $\alpha_v\beta_3$  acts as a pivotal regulator of angiogenesis. However, recent studies have raised questions challenging this view (61). Human or mice deficient in the integrin  $\beta_3$  subunit display normal developmental angiogenesis and are viable and fertile, although they have a bleeding disorder due to defects in the platelet integrin  $\alpha_{IIb}\beta_3$  (62). This finding indicates that  $\alpha_v\beta_3$  is not absolutely required for developmental angiogenesis. Furthermore, mice with targeted disruptions in integrin  $\beta_3$  or both  $\beta_3$  and  $\beta_5$  subunits grow larger tumors than wild type, suggesting that integrins  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  may actually be negative regulators of angiogenesis (63). These observations indicate that the roles of  $\alpha_v$  integrins in angiogenesis may be more complex than

previously thought. Nevertheless, the ability of CCN3 to bind integrin  $\alpha_v\beta_3$  and to promote endothelial cell adhesion and migration through this integrin shows that angiogenic actions may be mediated through  $\alpha_v\beta_3$ . In this context, it is possible to contemplate the actions of CCN3 both as an inducer via direct binding to  $\alpha_v\beta_3$ , and as a modulator by competition with other ligands that bind integrin  $\alpha_v\beta_3$ .

[0032] Members of the CCN family of matricellular proteins clearly serve important developmental functions. Deficiency in CCN6 causes progressive pseudorheumatoid dysplasia in humans, a juvenile-onset degenerative disease of the cartilage (64). Although both CCN1 and CCN2 are angiogenic inducers, mutations in their structural genes result in distinct phenotypes related to angiogenic defects. Targeted gene disruption of *CCN1* in mice resulted in embryonic lethality with vascular defects in both the placenta and the embryo (23). *CCN2*-null mice, on the other hand, are perinatal lethal as a consequence of respiratory failure due to skeletal malformations (18). Interestingly, angiogenic defects in the growth plates underlie part of the skeletal defects in *CCN2* mutants. These findings indicate that although proteins of the CCN family share extensive sequence homology and overlapping activities *in vitro*, they serve non-redundant developmental roles. Thus, although CCN3 induces angiogenesis, its biological functions may be distinct from those of CCN1 and CCN2. The observation that CCN3 is the only CCN protein known to bind integrin  $\alpha_5\beta_1$  also support the notion that CCN3 has unique functions.

### 1. Screening for Modulators of CCN3

[0033] The present invention involves screening for modulators of activities associated with CCN3. Modulators may be identified that directly bind to CCN3, thereby prevent CCN3 from

interacting with target proteins. Modulators may also be identified that directly bind to targets proteins of CCN3, thereby preventing CCN3 from productively interacting with said target protein. Modulators may also be identified which indirectly affect binding of CCN3 to target proteins.

[0034] For purposes of the invention, an "ECM signaling molecule" refers to CCN3 or a fragment, variant, analog, homolog or a derivative thereof that maintains as least one biological activity of CCN3. The use of "ECM signaling molecule" also contemplates one or more additional CCN polypeptides. The one or more additional CCN polypeptides include, but are not limited to, CCN1, CCN2, CCN4, CCN5 and CCN6, as well as fragments, variants, analogs, homologs or derivatives of said one or more additional CCN polypeptides.

**a. Angiogenesis**

[0035] The methods of the present invention relate to screening for a modulator of angiogenesis. In one embodiment of the present invention, a biological sample capable of undergoing angiogenesis is contacted with a suspected modulator *in vitro* along with an ECM signaling molecule. As a control, a second biological sample is also contacted with an ECM signaling molecule. A modulator of angiogenesis may be identified by its ability to alter the level of angiogenesis of the test sample when compared to the control sample.

[0036] In another embodiment of the present invention, an implant comprising a suspected modulator and an ECM signaling molecule is implanted into a test animal. As a control, a second implant comprising an ECM signaling molecule is implanted into a test animal, which may be the same animal or a different test animal. A modulator of angiogenesis may be identified by its ability to alter the level of blood vessel development in the test implant when compared to the control sample.

**b. Oncogenesis**

[0037] The methods of the present invention also relate to screening for a modulator of oncogenesis. A tumor is contacted with a suspected modulator along with an ECM signaling molecule. As a control, a second tumor is also contacted with an ECM signaling molecule. A modulator of oncogenesis may be identified by its ability to alter the level of oncogenesis of the test tumor when compared to the control tumor.

**c. Cell Adhesion**

[0038] The methods of the present invention also relate to screening for a modulator of cell adhesion. A biological sample on a surface compatible with cell adherence is contacted with a suspected modulator along with an ECM signaling molecule. As a control, a second biological sample on a surface compatible with cell adherence is also contacted with an ECM signaling molecule. A modulator of cell adhesion may be identified by its ability to alter the level of cell adhesion of the test sample when compared to the control sample.

**d. Cell Migration**

[0039] The methods of the present invention also relate to screening for a modulator of cell migration. Cells capable of undergoing cell migration are seeded onto a gel matrix comprising a suspected modulator and an ECM signaling molecule. As a control, cells capable of undergoing cell migration are also seeded onto a second biological sample gel matrix comprising an ECM signaling molecule. A modulator of cell adhesion may be identified by its ability to alter the level of cell migration in the test matrix when compared to the control matrix.

**e. Identified Modulators**

[0040] The present invention also involves modulators of CCN3 activity identified using the above-described screening methods. The identified modulators of CCN3 activity may be

formulated in a pharmaceutical composition comprising a pharmaceutically acceptable adjuvant, diluent, or carrier. The pharmaceutical composition comprising the modulator of CCN3 activity may be administered to a patient for the treatment of disease associated with

## 2. Inhibitory Peptides

[0041] The present invention also involves the use of inhibitory peptides in therapeutic strategies designed to inhibit the activity of CCN3. The inhibitory peptides may be natural, synthetic or recombinant. One approach is to produce an inhibitory peptide based on the protein sequence of CCN3 (SEQ ID NO: 1). For example, a peptide comprising conserved amino acids may compete with native CCN3 for its binding sites. This competition may thereby inhibit the action of native CCN3. The inhibitory peptides may be from 5 to 50 amino acids in length. The inhibitory peptides may be 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 amino acids in length.

[0042] The inhibitory peptide may comprise amino acids from SEQ ID NO: 1 selected from the group consisting of 1-5, 3-7, 6-10, 8-12, 11-15, 13-17, 16-20, 18-22, 21-25, 23-27, 26-30, 28-32, 31-35, 33-37, 36-40, 38-42, 41-45, 43-47, 46-50, 48-52, 51-55, 53-57, 56-60, 58-62, 61-65, 63-67, 66-70, 68-72, 71-75, 73-77, 76-80, 78-82, 81-85, 83-87, 86-90, 88-92, 91-95, 93-97, 96-100, 98-102, 101-105, 103-107, 106-110, 108-112, 111-115, 113-117, 116-120, 118-122, 121-125, 123-127, 126-130, 128-132, 131-135, 133-137, 136-140, 138-142, 141-145, 143-147, 146-150, 148-152, 151-155, 153-157, 156-160, 158-162, 161-165, 163-167, 166-170, 168-172, 171-175, 173-177, 176-180, 178-182, 181-185, 183-187, 186-190, 188-192, 191-195, 193-197, 196-200, 198-202, 201-205, 203-207, 206-210, 208-212, 211-215, 213-217, 216-220, 218-222, 221-225, 223-227, 226-230, 228-232, 231-235, 233-237, 236-240, 238-242, 241-245, 243-247, 246-250,



248-252, 251-255, 253-257, 256-260, 258-262, 261-265, 263-267, 266-270, 268-272, 271-275, 273-277, 276-280, 278-282, 281-285, 283-287, 286-290, 288-292, 291-295, 293-297, 296-300, 298-302, 301-305, 303-307, 306-310, 308-312, 311-315, 313-317, 316-320, 318-322, 321-325, 323-327, 326-330, 328-332, 331-335, 333-337, 336-340, 338-342, 341-345, 343-347, 346-350, 348-352, 351-355, 353-357.

[0043] The inhibitory peptide may also comprise amino acids from SEQ ID NO: 1 selected from the group consisting of 1-10, 6-19, 11-20, 16-29, 21-30, 26-39, 31-40, 36-49, 41-50, 46-59, 51-60, 56-69, 61-70, 66-79, 71-80, 76-89, 81-90, 86-99, 91-100, 96-109, 101-110, 106-119, 111-120, 116-129, 121-130, 126-139, 131-140, 136-149, 141-150, 146-159, 151-160, 156-169, 161-170, 166-179, 171-180, 176-189, 181-190, 186-199, 191-200, 196-209, 201-210, 206-219, 211-220, 216-229, 221-230, 226-239, 231-240, 236-249, 241-250, 246-259, 251-260, 256-269, 261-270, 266-279, 271-280, 276-289, 281-290, 286-299, 291-300, 296-309, 301-310, 306-319, 311-320, 316-329, 321-330, 326-339, 331-340, 336-349, 341-350, 346-357.

[0044] The inhibitory peptide may also comprise amino acids from SEQ ID NO: 1 selected from the group consisting of 1-15, 8-22, 16-30, 23-37, 31-45, 38-52, 46-60, 53-67, 61-75, 68-82, 76-90, 83-97, 91-105, 98-112, 106-120, 113-127, 121-135, 128-142, 136-150, 143-157, 151-165, 158-172, 166-180, 173-187, 181-195, 188-202, 196-210, 203-217, 211-225, 218-232, 226-240, 233-247, 241-255, 248-262, 256-270, 263-277, 271-285, 278-292, 286-300, 293-307, 301-315, 308-322, 316-330, 323-337, 331-345, 338-352, 346-357.

[0045] The inhibitory peptides may also be homologs of the above-described CCN3 peptides. Homologs of the CCN3 peptides are peptides sharing a common evolutionary with SEQ ID NO: 1. The inhibitory peptides may also be variants of the above-described CCN3 peptides and homologs. Inhibitory peptide variants are peptides that differ in amino acid sequence from a

native CCN3 peptide by the insertion, deletion, or conservative substitution of amino acids, but retain at least one biological activity of a native CCN3 peptide. For purposes of the present invention, "biological activity of a CCN3 peptide" includes, but is not limited to, the above-described activities of full-length CCN3, the ability to inhibit activities of CCN3 and the ability to be bound by an antibody specific for CCN3.

[0046] A conservative substitution of an amino acid, *i.e.*, replacing an amino acid with a different amino acid of similar properties (*e.g.*, hydrophilicity, degree and distribution of charged regions) is recognized in the art as typically involving a minor change. These minor changes can be identified, in part, by considering the hydropathic index of amino acids, as understood in the art. *Kyte et al., J. Mol. Biol. 157:105-132 (1982)*. The hydropathic index of an amino acid is based on a consideration of its hydrophobicity and charge, and include the following values: alanine (+1.8), arginine (-4.5), asparagine (-3.5), aspartate (-3.5), cysteine/cysteine (+2.5), glycine (-0.4), glutamate (-3.5), glutamine (-3.5), histidine (-3.2), isoleucine (+4.5), leucine (+3.8), lysine (-3.9), methionine (+1.9), phenylalanine (+2.8), proline (-1.6), serine (-0.8), threonine (-0.7), tryptophan (-0.9), tyrosine (-1.3), and valine (+4.2). It is known in the art that amino acids of similar hydropathic indexes can be substituted and still retain protein function. In one aspect, amino acids having hydropathic indexes of  $\pm 2$  are substituted.

[0047] The hydrophilicity of amino acids can also be used to reveal substitutions that would result in proteins retaining biological function. A consideration of the hydrophilicity of amino acids in the context of a peptide permits calculation of the greatest local average hydrophilicity of that peptide, a useful measure that has been reported to correlate well with antigenicity and immunogenicity. U.S. Patent No. 4,554,101, incorporated herein by reference. Hydrophilicity values for each of the common amino acids, as reported in U.S. Patent No. 4,554,101, are:

alanine (-0.5), arginine (+3.0), asparagine (+0.2), aspartate (+3.0  $\pm$  1), cysteine (-1.0), glycine (0), glutamate (+3.0  $\pm$  1), glutamine (+0.2), histidine (-0.5), isoleucine (-1.8), leucine (-1.8), lysine (+3.0), methionine (-1.3), phenylalanine (-2.5), proline (-0.5  $\pm$  1), serine (+0.3), threonine (-0.4), tryptophan (-3.4), tyrosine (-2.3), and valine (-1.5). Substitution of amino acids having similar hydrophilicity values can result in peptides retaining biological activity, for example immunogenicity, as is understood in the art. In one aspect, substitutions are performed with amino acids having hydrophilicity values within  $\pm$  2 of each other. Both the hydrophobicity index and the hydrophilicity value of amino acids are influenced by the particular side chain of that amino acid. Consistent with that observation, amino acid substitutions that are compatible with biological function are understood to depend on the relative similarity of the amino acids, and particularly the side chains of those amino acids, as revealed by the hydrophobicity, hydrophilicity, charge, size, and other properties.

[0048] Additionally, computerized algorithms are available to assist in predicting amino acid sequence domains likely to be accessible to an aqueous solvent. These domains are known in the art to frequently be disposed towards the exterior of a peptide, thereby potentially contributing to binding determinants, including antigenic determinants.

[0049] The inhibitory peptides may also be analogs of the above-described CCN3 peptides, homologs and variants comprising non-standard amino acid or other structural variation from the conventional set of amino acids. The inhibitory peptides may also be derivatives of the above-described CCN3 peptides, homologs, variants and analogs that differ in ways other than primary structure (amino acids and amino acid analogs). By way of illustration, derivatives may differ from native CCN3 peptides, homologs and variants by being glycosylated, one form of post-translational modification. For example, polypeptides may exhibit glycosylation patterns

due to expression in heterologous systems. If these peptides retain at least one biological activity of native CCN3, then these peptides are CCN3 derivatives according to the invention. Other derivatives include, but are not limited to, fusion peptides having a covalently modified N- or C-terminus, PEGylated peptides, peptides associated with lipid moieties, alkylated peptides, peptides linked via an amino acid side-chain functional group to other peptides or chemicals, and additional modifications as would be understood in the art. In addition, the invention contemplates CCN3-related peptides that bind to a CCN3 receptor, as described below.

[0050] The various peptides of the present invention, as described above, may be provided as discrete peptides or be linked, *e.g.*, by covalent bonds, to other compounds. For example, immunogenic carriers such as Keyhole Limpet Hemocyanin may be bound to a CCN3 peptide of the invention.

### 3. Antibodies

[0051] The present invention also involves a pharmaceutical composition comprising an antibody that specifically binds to CCN3 and a pharmaceutically acceptable adjuvant, diluent, or carrier. The antibody may be produced as described below, or as described in WO 01/55210, the contents of which are hereby incorporated by reference in their entirety.

[0052] The antibodies of the present invention include antibodies of classes IgG, IgM, IgA, IgD, and IgE, and fragments and derivatives thereof including Fab and F(ab')<sub>2</sub>. The antibodies may also be recombinant antibody products including, but not limited to, single chain antibodies, chimeric antibody products, "humanized" antibody products, and CDR-grafted antibody products. The antibodies of the present invention include monoclonal antibodies, polyclonal antibodies, affinity purified antibodies, or mixtures thereof which exhibit sufficient binding specificity to CCN3.

[0053] Also contemplated by the invention are antibody fragments. The antibody products include the aforementioned types of antibody products used as isolated antibodies or as antibodies attached to labels. Labels can be signal-generating enzymes, antigens, other antibodies, lectins, carbohydrates, biotin, avidin, radioisotopes, toxins, heavy metals, and other compositions known in the art; attachment techniques are also well known in the art.

[0054] Anti-CCN3 antibodies are useful in diagnosing the risk of oncogenesis. In addition, anti-CCN3 antibodies are used in therapies designed to deliver specifically-targeted cytotoxins to cells expressing CCN3, *e.g.*, cells participating in the neovascularization of solid tumors. These antibodies are delivered by a variety of administrative routes, in pharmaceutical compositions comprising carriers or diluents, as would be understood by one of skill in the art.

[0055] The present invention has multiple aspects, illustrated by the following non-limiting examples. Example 1 describes the cloning, expression and purification of recombinant CCN3. Example 2 describes the production of anti-CCN3 antibodies. Example 3 discloses that CCN3 supports endothelial cell adhesion. Example 4 discloses that CCN3 acts as a ligand of integrin receptors. Example 5 discloses that CCN3 directs cells migration. Example 6 discloses that CCN3 promotes cell survival. Example 7 demonstrates that induces neovascularization *in vivo*. These examples are intended to be illustrative of the present invention and should not be construed to limit the scope of the invention.

## EXAMPLE 1

### Purification of Recombinant CCN3

[0056] Human *CCN3* cDNA was constructed by ligation of a 5' (nt 72-654, Genbank X96584) and a 3' (nt 654-1653) fragments, and the resulting full-length cDNA was cloned into pKS+ and

verified by sequencing. The 5' fragment (nt 72-654) was obtained by reverse transcriptase-polymerase chain reaction using total RNA isolated from serum-starved human skin fibroblasts using the primer set 5'-AGCAGTGCCAATCTACAGC-3' and 5'-CAGCATCTCACATTGACGG-3'. The RT-PCR product was digested with SphI and StyI to yield a fragment containing nt 72-654. The 3' fragment (nt 654-1653) was generated by restriction digestion of IMAGE clone #49415 (human neonatal brain, nt 590-1653) with StyI and XbaI. To produce recombinant CCN3 protein, the full-length *CCN3* cDNA was cloned into the baculovirus expression vector pBlueBac 4.5 (Invitrogen, Carlsbad, CA). The vector was modified to encode an enterokinase histidine tag linked to the C-terminus of CCN3 in a manner similar to that previously described for expression of CCN1 (21).

[0057] CCN3 was produced in serum-free baculovirus expression system using High Five insect cells as described (21). Briefly, High Five cells were maintained in serum-free EX-CELL 400 medium (JRH Bioscience, Lenexa, KS) at 27°C and infected at a multiplicity of infection of 10. Conditioned medium was collected at 38 h post-infection, adjusted to 20 mM sodium phosphate and applied to a Sepharose SP (Sigma-Aldrich, St. Louis, MO) column at 4°C. After washing with a 20 mM sodium phosphate buffer (pH 6.0) containing 300 mM NaCl, bound proteins were eluted with a linear gradient of NaCl (0.4 - 1 M) in phosphate buffer. Fractions containing CCN3 as judged by SDS-PAGE were pooled and further purified on a nickel-agarose column as described (21). Fractions were analyzed by SDS-PAGE followed by Coomassie brilliant blue staining and immunoblotting (Fig. 1).

## EXAMPLE 2

### Preparation of anti-CCN3 Antibodies

[0058] The second domain of CCN3 (von Willebrand type C repeat) and the central variable region were cloned separately as glutathione S-transferase (GST) fusion proteins and used as antigens to immunize New Zealand white rabbits. DNA fragments were generated by polymerase chain reactions using primers sets 5'-CGCGGATCCGCGGTAGAGGGAGATAACTGTG-3' and 5'-CCGGAATTCAGCTGCAAGGGTAAGGCCTCC-3' (encoding a.a. 104-188), and 5'-GATGAGGAGGATTCACTGGGA-3 and 5'-AATGCAGTTGACACTTGAG-3' (encoding a.a. 176-207). To facilitate cloning, the forward primers start with a BamHI site and the reverse primers end with an EcoRI site. The resulting cDNA fragments were cloned directionally into the PGEX-2T vector (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) and confirmed by sequence analysis. The GST-fusion proteins were purified on a glutathione-S sepharose column and used as antigens.

[0059] Antisera and affinity-purified antibodies were produced according to standard protocol (40). IgG was purified from antisera using protein A column chromatography (Pierce Biotechnology, Rockford, IL). For affinity purification, antisera were first passed through a GST column to remove antibodies against GST, and then purified through a GST-CCN3 (VWC domain)-affinity column. Anti-CCN3 antibodies did not cross react with CCN1 or VN (data not shown) by ELISA.

### EXAMPLE 3

#### Cell Adhesion

[0060] To examine the proangiogenic activities of CCN3, purified recombinant human CCN3 was tested for the ability to support endothelial cell adhesion. Cell adhesion assays were performed essentially as described (7). Briefly, test proteins were diluted in PBS and coated onto 96-well microtiter plates (50  $\mu$ l per well) with incubation at 4°C for 16 h. Wells were rinsed with PBS and blocked with 1% BSA at RT for 1 h. To test for specificity, affinity-purified anti-CCN3 antibodies or normal rabbit IgG was added to the wells and incubated for 1 h at 37°C prior to plating of cells.

[0061] HUVECs were cell cultured as described by the supplier (Cascade Biologics, Inc., Portland, OR) and used before passage 16. HUVECs were harvested in PBS containing 2.5 mM EDTA, washed and resuspended at  $2.5 \times 10^5$  cells/ml in serum-free Iscove's modified Dulbecco's medium containing 1% BSA. Where indicated, cells were mixed with either EDTA,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , peptides, or heparin prior to plating or incubated with antibodies for 1 h at RT prior to plating. Cell suspension (50  $\mu$ l) was added to each well, and adherent cells were fixed in 10% formalin after 30 min incubation at 37°C. Cells were stained with methylene blue, and adhesion was quantified by dye extraction and measurement of absorbance at 620 nm (7).

[0062] Immobilized CCN3 was able to support HUVEC adhesion in a dose-dependent and saturable manner (Fig 2A). Incubation of CCN3-coated surfaces with affinity-purified anti-CCN3 antibodies prior to plating cells abolished adhesion, whereas incubation of VN-coated surfaces with the same antibodies had no effect (Fig 2B). These results confirmed that the CCN3 protein indeed supports HUVEC adhesion.



[0063] CCN1 and CCN2 are known to mediate HUVEC adhesion through integrin  $\alpha_v\beta_3$  (19) and fibroblast adhesion through integrin  $\alpha_6\beta_1$  and heparin sulfate proteoglycans (8,20). HUVEC adhesion to CCN3, as well as to FN or VN, was inhibited by EDTA and restored by the addition of  $Mg^{2+}$  or  $Ca^{2+}$  (Fig. 2C). These results are consistent with the notion that divalent cation-dependent cell adhesion molecules, such as integrins, mediate cell adhesion to CCN3 (Fig. 2C).

[0064] To define the specific integrins that might be involved, we investigated the inhibitory effect of RGD peptide. The GRGDSP peptide (Invitrogen-Carlsbad, CA), but not the control GRGESp peptide (Invitrogen), was able to partially block cell adhesion to CCN3 and completely block adhesion to VN (Invitrogen) (Fig. 2D), indicating the involvement of RGD-sensitive integrins. Since integrin  $\alpha_v\beta_3$  (Chemicon-Temecula, CA) is known to be inhibited by a relatively low concentration of RGD peptide (0.2 mM) as shown in Fig. 2D, we examined the role of this integrin. Cells were incubated with LM609 (Chemicon), a mAb against integrin  $\alpha_v\beta_3$ , prior to plating. LM609 partially blocked HUVEC adhesion to CCN3 and VN but not to FN (Invitrogen) (Fig 3B). We also tested the possible involvement of integrin  $\alpha_5\beta_1$  (Chemicon) given its RGD sensitivity, even though no other CCN protein is known to bind this integrin. Surprisingly, mAb against integrin  $\alpha_5\beta_1$  (JSB5) (Chemicon) was also able to partially block HUVEC adhesion to CCN3 and FN (Fig. 3C). As expected, the same mAb had no effect on HUVEC adhesion to VN. These results indicate that both integrins  $\alpha_5\beta_1$  and  $\alpha_v\beta_3$  play a role in HUVEC adhesion to CCN3.

[0065] Since it was previously shown that CCN1 and CCN2 mediate adhesion of fibroblasts through integrin  $\alpha_6\beta_1$  and heparin sulfate proteoglycans acting as co-receptors (8,20), we investigated whether these two receptors also mediate HUVEC adhesion to CCN3. To investigate the potential role of integrin  $\alpha_6\beta_1$ , cells were incubated with mAbs against either

integrin  $\alpha_6$  (GoH3) (Beckman-Coulter, Inc.-Fullerton, CA) or  $\beta_1$  (P4C10) (Invitrogen-Carlsbad, CA) subunit prior to plating. These mAbs partially blocked adhesion to CCN3, indicating that integrin  $\alpha_6\beta_1$  also plays a role in HUVEC adhesion to CCN3 (Fig. 3A). As expected, these mAbs partially blocked cell adhesion to LN (Invitrogen), which binds integrins  $\alpha_6\beta_1$  and  $\alpha_2\beta_1$ , but not to VN, which binds  $\alpha_v$  integrins. Soluble heparin is known to block fibroblast adhesion to CCN1 and CCN2 by saturating the heparin binding sites located in the CT domain, thereby preventing them from binding cell surface heparin sulfate proteoglycans (8,20). Likewise, soluble heparin (Sigma-Aldrich-St. Louis, MO) partially blocked HUVEC adhesion to CCN3, but not to FN or VN (Fig. 2D). Thus, it is likely that CCN3 may also engage heparin sulfate proteoglycans as a co-receptor when interacting with integrin  $\alpha_6\beta_1$ .

[0066] Given that antagonists of integrins  $\alpha_v\beta_3$ ,  $\alpha_6\beta_1$ , and  $\alpha_5\beta_1$  were able to partially inhibit HUVEC adhesion to CCN3, we tested whether the combination of antagonists of these integrins were sufficient to account for endothelial cell adhesion to CCN3. When cells were treated with GRGDSP peptide and soluble heparin, CCN3 adhesion was obliterated (Fig 2D). In contrast, adhesion of the same cells to FN was not affected. Also, when HUVECs were incubated with function-blocking mAbs against integrins  $\alpha_v\beta_3$ ,  $\alpha_6$ , and  $\alpha_5\beta_1$ , cell adhesion to CCN3 was completely abolished (Fig 3D). As expected, HUVEC adhesion to FN, VN and laminin was only partially blocked by these antibodies. Together, these results indicate that endothelial cell adhesion to CCN3 is mediated through the combined actions of integrins  $\alpha_v\beta_3$ ,  $\alpha_5\beta_1$ , and  $\alpha_6\beta_1$ .

## EXAMPLE 4

### Binding of CCN3 to Integrins

[0067] Since CCN3 mediates HUVEC adhesion through integrins  $\alpha_v\beta_3$ ,  $\alpha_5\beta_1$  and  $\alpha_6\beta_1$  (Fig. 3D), we investigated whether CCN3 can bind integrin receptors directly using ELISA similar to as previously described. For CCN3 binding to immobilized integrin, microtiter wells (Immulon 2, Dynatech Laboratories, Chantilly, VA) were coated with purified integrin (1  $\mu\text{g/ml}$ ) and incubated at 4°C overnight. The wells were washed with buffer (20 mM Hepes, pH 7.5 containing 150 mM NaCl, 0.1 mM  $\text{CaCl}_2$ , and 2 mM  $\text{MgCl}_2$ ) and blocked with 1% heat-inactivated BSA for 2 h at RT. Soluble CCN3 was added and allowed to bind at 4°C for 16 h; bound ligand was detected using affinity-purified anti-CCN3 antibodies (1:1000). For integrin binding to immobilized ligands, microtiter wells were coated with 10  $\mu\text{g/ml}$  CCN3, 10  $\mu\text{g/ml}$  FN or 1  $\mu\text{g/ml}$  VN as described above. Where indicated, coated wells were pre-incubated with affinity-purified anti-CCN3 antibodies or normal rabbit serum for 2 h at RT. After washing, purified integrin  $\alpha_v\beta_3$  or  $\alpha_5\beta_1$  (1  $\mu\text{g/ml}$  in buffer with 25 mM octylglucoside) was added and incubated overnight at 4°C. Where indicated, soluble integrin was either mixed with EDTA,  $\text{Mg}^{2+}$ , or peptides prior to plating, or incubated with function-blocking monoclonal antibodies for 30 min at 4°C prior to plating. After washing, bound integrins were detected with polyclonal anti-integrin  $\alpha_v$  (AB1930) or  $\alpha_5$  antibodies (AB1926)(Chemicon, Temecula, CA). When blocking with anti-CCN3 antibodies, mAbs against integrin  $\alpha_v$  (P3G8) or  $\beta_1$  (HUTS-4) (Chemicon, Temecula, CA) were used to detect integrin binding. After washing, wells were incubated with horseradish peroxidase-conjugated secondary antibody (1:2500), and color

reaction was developed using a horseradish peroxidase immunoassay kit (Zymed Laboratories, Inc., South San Francisco, CA) with absorbance measured at 420 nm.

[0068] As shown in Figs. 4A and 5A, CCN3 was able to bind immobilized integrins  $\alpha_v\beta_3$  and  $\alpha_5\beta_1$  in a dose-dependent and saturable manner, with half maximal binding occurring at 0.4  $\mu\text{g/ml}$  (10 nM) and 1  $\mu\text{g/ml}$  (50 nM) CCN3, respectively. Conversely, CCN3 was immobilized on microtiter wells and allowed to interact with integrins  $\alpha_v\beta_3$  or  $\alpha_5\beta_1$ . Binding of integrins to immobilized CCN3 was observed using antibodies against integrin  $\alpha_v$  or  $\alpha_5$  subunits (Figs. 4B-D, 5B-D).

[0069] We employed a variety of antagonists to address the specificity of the interaction between CCN3 and integrins  $\alpha_v\beta_3$  and  $\alpha_5\beta_1$ . When CCN3 was incubated with affinity-purified anti-CCN3 polyclonal antibodies, binding to integrins  $\alpha_v\beta_3$  and  $\alpha_5\beta_1$  was abolished (Fig. 4B, 5B). By contrast, incubation of FN (Fig. 5B) or VN (Fig. 4B) with the same antibodies had no effect, as expected. Divalent cations are required for integrin function, and both integrins  $\alpha_v\beta_3$  and  $\alpha_5\beta_1$  can be inhibited by RGD-containing peptides (41). As shown in Figs. 4C and 5C, EDTA completely abrogated the binding of integrins  $\alpha_v\beta_3$  and  $\alpha_5\beta_1$  to CCN3, as well as to VN and FN. As expected, binding was restored upon the addition of  $\text{MgCl}_2$ . RGDS peptide, but not RGES peptide, was able to inhibit binding of integrins  $\alpha_v\beta_3$  and  $\alpha_5\beta_1$  to CCN3 and to positive controls, VN or FN. Furthermore, mAb against integrin  $\alpha_v\beta_3$  (LM609) blocked binding of integrin  $\alpha_v\beta_3$  to CCN3 and VN (Fig. 4D), and mAb against integrin  $\alpha_5\beta_1$  (JBS5) inhibited binding of integrin  $\alpha_5\beta_1$  to CCN3 and FN. Taken together, these results show that CCN3 is a novel ligand of integrins and binds directly and specifically to integrins  $\alpha_v\beta_3$  and  $\alpha_5\beta_1$ .

## EXAMPLE 5

### Cell Migration

[0070] Cell migration is an integral part of the angiogenic process, and induction of endothelial cell chemotaxis correlates with angiogenic activity (42). We thus investigated whether CCN3 can stimulate migration of endothelial cells using a modified Boyden chamber assay. A 48-well modified Boyden chamber (Neuro Probe, Inc., Gaithersburg, MD) was used to assay cell migration as described with modifications (16). BACECs (a generous gift of Dr. Judah Folkman, Harvard Medical School) were grown in plates coated with 1.5 % gelatin (Difco Laboratories, Detroit, MI) and maintained in Dulbecco's modified Eagle's medium (DMEM, JRH Bioscience, Lenexa, KS) supplemented with 10 % fetal bovine serum (Intergene, Purchase, NY) and 3 ng/ml bFGF (Invitrogen, Carlsbad, CA); they were used before passage 16.

[0071] BACECs were harvested with trypsin, washed and resuspended at  $5 \times 10^5$  cells/ml in DMEM containing 0.1% BSA. Cells were loaded into wells of the lower chamber; the wells were then covered with a gelatinized polycarbonate filter (5  $\mu$ m pore diameter, Nuclepore, Newton, MA) followed by the upper chamber. Where indicated, cells were either mixed with peptides or incubated with antibodies (1h at RT) prior to loading. The chamber was inverted to allow cells to attach (2 h at 37°C) and re-inverted and test proteins, diluted in DMEM containing 0.1% BSA, were added to the top chamber. Where indicated, CCN3 and FN were pre-incubated with anti-CCN3 antibodies for 1 h prior to loading. After a 4 h incubation at 37°C, the membrane was removed and stained using a Diff-Quik Kit (Dade-Behring, Deerfield, IL). Cell migration was monitored by counting the total number of cells migrated in 10 randomly selected microscope fields at 400 X magnification.

[0072] CCN3 was able to stimulate migration of BACECs (Fig. 6A). CCN3-induced migration was dose-dependent and reached maximal level at 0.25  $\mu\text{g/ml}$ . Incubation of CCN3 protein with affinity-purified anti-CCN3 antibodies abolished CCN3-induced migration, while incubation with the same antibodies did not affect FN-stimulated migration (Fig. 6B), indicating that cell migration can be attributed to CCN3.

[0073] Stimulation of cell migration can be due to a chemotactic (directed cell movement) or a chemokinetic (random cell movement) response. In order to determine whether CCN3 induces chemotaxis or chemokinesis of BACECs, a checkerboard analysis was performed. CCN3 was placed in the upper chamber (no cells), in the lower chamber (with cells), and in both or neither chambers (Fig. 6C). Addition of CCN3 to the lower chamber did not enhance BACEC migration to the upper chamber, indicating that CCN3 did not induce a chemokinetic response. Addition of CCN3 to the upper chamber induced the maximal level of migration, consistent with a chemotaxis. Addition of CCN3 to both chambers reduced the level of BACEC migration, suggesting that BACECs are sensitive to a CCN3 gradient. Together, these results show that CCN3 induces directed endothelial cell migration.

[0074] Since CCN3 was shown to be a ligand for integrins  $\alpha_v\beta_3$  and  $\alpha_5\beta_1$ , both of which can mediate cell migration, we tested whether CCN3 promotes BACEC migration through these integrins. Incubation of cells with GRGDSP peptide completely inhibited migration to CCN3 and VN (Fig. 6D). No effect on migration was observed with cells incubated with GRGESp control peptide. Consistent with the involvement of integrin  $\alpha_v\beta_3$ , anti-integrin  $\alpha_v$  mAbs (AV1) (Chemicon) and anti-integrin  $\alpha_v\beta_3$  mAbs (LM609) partially block BACEC migration to CCN3 (Fig. 7A). As expected, these antibodies inhibited BACEC migration to VN but had no effect on cell migration to FN. In addition, anti-integrin  $\alpha_5$  mAb (SAM-1) (Beckman-Coulter, Inc.)

partially inhibited CCN3-stimulated cell migration but not VN-stimulated cell migration (Fig. 7B), indicating that integrin  $\alpha_5\beta_1$  may also be involved. Moreover, a combination of mAbs against integrin  $\alpha_v\beta_3$  and  $\alpha_5$  mAb (LM609 and SAM-1) completely abolished BACEC migration to CCN3. As expected, these mAbs partially inhibited cell migration to VN and FN and had no effect on cell migration to laminin (Fig. 7C). To address whether integrin  $\alpha_6\beta_1$  might also play a role, we examined the inhibitory activity of the anti- $\alpha_6$  mAb, GoH3 (Fig. 7D). BACEC migration to CCN3 or FN was unaffected by the presence of GoH3, whereas cell migration to laminin was partially inhibited. Taken together, these results show that of the three integrins known to interact with CCN3 (Fig. 2-5), integrins  $\alpha_v\beta_3$  and  $\alpha_5\beta_1$  mediate endothelial cell migration to CCN3, whereas integrin  $\alpha_6\beta_1$  is not involved in this process.

## EXAMPLE 6

### Cell Survival

[0075] During angiogenesis, endothelial cells require survival signals in order to migrate, proliferate and interact with the remodeling ECM. Ligation of integrin  $\alpha_v\beta_3$  has been shown to induce survival signals in endothelial cells (43). To investigate the possibility that CCN3 may promote cell survival, apoptosis and mitogenesis were assessed largely as described (14). To measure apoptosis, HUVECs were starved for 16 h, harvested and resuspended in serum free medium containing 0.5% BSA. Coverslips were coated overnight at 4°C with 20  $\mu$ g/ml mouse LN (ultrapure grade; Becton-Dickinson Biosciences, Bedford, MA) and blocked with 1% heat-inactivated BSA. Cells were plated on LN-coated coverslips at 10,000 cells/cm<sup>2</sup> and allowed to attach for 4 h at 37°C. Soluble CCN3, serum (20%) or GRGDSP peptide (50  $\mu$ M) was added and incubation continued for another 16 h at 37°C. Where indicated, CCN3 was incubated with

anti-CCN3 antibodies for 1 h at RT prior to addition to cells. After incubation, cells were fixed with 4% paraformaldehyde (pH 7.4) and apoptosis was detected by TUNEL assay using the *in situ* cell death detection kit POD (Roche Applied Science, Indianapolis, IN). Cells were lightly stained with hematoxylin and apoptotic nuclei counted. A total of 500 cells were counted from random fields in each coverslip, and the number of apoptotic cells was represented as a percentage of the total cells counted. To assess proliferation, cells were grown as described above except that 10  $\mu$ M bromodeoxyuridine (BrdUrd) was included in the medium for 16 h in the presence or absence of CCN3. BrdU incorporation was detected using the BrdUrd staining kit (Calbiochem Novabiochem Corp., San Diego, CA).

[0076] HUVECs were plated on laminin and maintained in serum-free medium. Under these conditions, endothelial cells were susceptible to apoptosis (44). GRGDSP peptide, 20% serum, or varying concentrations of CCN3 were then added to cells, and apoptosis was determined using a TUNEL assay after 16 hrs (Fig. 8A). Under these conditions, CCN3 was able to promote endothelial cell survival in a dose-dependent manner. Serum also protected cells from apoptosis, while GRGDSP peptide promoted apoptosis. Pre-incubation of CCN3 with affinity-purified anti-CCN3 antibodies abolished CCN3-promoted cell survival. This was not due to antibody toxicity since addition of antibodies alone did not affect cell survival. Taken together, these results show that CCN3 can protect endothelial cells from apoptosis and promote survival. To rule out the possibility that an increased number of non-apoptotic cells might be due to cell proliferation, HUVECs were treated in the same manner as described above and proliferation was measured using BrdUrd incorporation (Fig. 8B). The rate of DNA synthesis was not affected by the presence or absence of CCN3. These results show that CCN3 is not a mitogen



for HUVECs under these conditions, and that CCN3 is able to promote endothelial cell survival under otherwise apoptotic conditions.

#### EXAMPLE 7

##### Cornea Assay

[0077] **CCN3 induces neovascularization *in vivo*.** The ability of CCN3 to promote endothelial cell adhesion, migration, and survival are consistent with properties of an angiogenic inducer. We therefore investigated whether CCN3 could induce angiogenesis *in vivo* using the rat corneal micropocket assay. CCN3-induced neovascularization was examined *in vivo* by implanting Hydron pellets, formulated with test substances, into rat corneas essentially as described (16). Briefly, male Sprague-Dawley rats were anesthetized and Hydron pellets (Interferon Sciences, Inc., New Brunswick, NJ) containing test substances were implanted into micropockets made in the corneal stroma 1 to 1.5 mm from the corneal limbus. Where indicated, CCN3 and bFGF were incubated with anti-CCN3 antibodies for 1h at RT prior to being incorporated into the Hydron pellet. 7 days post implantation, rats were perfused with India ink with heparin (100-U bolus) and neovascularization was examined and scored.

[0078] As shown in Fig. 9, CCN3 was able to induce neovascularization when implanted into rat cornea, whereas the vehicle did not induce any response (Table 1). Neovascularization was also observed in corneas implanted with Hydron pellets containing bFGF, a known potent angiogenic inducer. Pre-incubation of CCN3 with anti-CCN3 antibodies obliterated CCN3-induced neovascularization, indicating that the angiogenic activities observed can be ascribed to the CCN3 polypeptide. Together, these results show that CCN3 can induce angiogenesis *in vivo*.

# <sup>1</sup>ABBREVIATIONS

[0079] BACEC, Bovine adrenal capillary endothelial cell; BSA, bovine serum albumin; CCN, cysteine-rich 61/connective tissue growth factor/nephroblastoma overexpressed; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; FN, fibronectin; GST, glutathione-S-transferase; HUVEC, human umbilical vein endothelial cell; IgG, immunoglobulin G; LN, laminin; mAb, monoclonal antibody; PBS, phosphate-buffered saline; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TUNEL, *in situ* terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; VN, vitronectin

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